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Multiyear Study of Sludge Application to Farmland: Prevalence of Bacterial Enteric Pathogens and Antibody Status of Farm Families

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16. ABSTRACT

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Multiyear Study of Sludge Application to Farmland: Prevalence of Bacterial Enteric Pathogens and Antibody Status of Farm Families

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We describe our experience with the isolation of salmonellae from sewage sludge from four treatment plants in different geographic areas of Ohio. Over 3 years, we isolated salmonellae 50 times from 311 sludge samples. Most isolations were made after enrichment in Selenite broth (BBL Microbiology Systems, Cockeysville, Md.). The largest proportion of isolations came from the plant serving the population of Columbus, a large metropolitan area. A significantly greater number of isolations from this plant were made during the first quarter of the year. Twenty-one different serotypes were isolated, along with five untypable strains. The most frequently isolated serotype was *Salmonella infantis*. Five of the strains were multiply resistant to antibiotics. We also describe the prevalence of antibodies to salmonellae in members of the families residing on the farms in the study. It was found that antibodies to group C salmonellae predominated.

As part of a study involving the application of wastewater sludge to farmlands in Ohio, the presence of bacteria and viruses known to cause enteric disease in humans was monitored. The study was designed to monitor farm workers and their families to assess the risk, if any, resulting from the application of wastewater sludge to land under controlled conditions. In this study, we describe the results of testing sludge samples for the presence of *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. We also describe the serological status of farm workers and their families at the beginning and end of the study. In addition, stool samples taken from the participants at regular intervals throughout the study were tested for the presence of *Salmonella* spp.

The epidemiological design is described in detail in the full project report (8). Briefly, farms in the study were assigned randomly either to receive sludge or to be controls, and the data were analyzed on the basis of matched farm pairs.

MATERIALS AND METHODS

Sewage plants and sludge samples. A total of 311 sludge samples were collected and tested for salmonellae and shigellae over 36 months. The samples were from four wastewater treatment plants in three geographic areas of Ohio. Details of the types of plants as well as of the physicochemical characteristics of the sludge produced at the different sites can be found in the complete project report (18) and have been summarized in a previous publication (13).

Sludges. Sludge samples were collected biweekly from the M300 and M500 plants in Medina County, the Jackson Pike Plant of Columbus, and the sewage treatment plant of the city of Springfield, Ohio. Samples of 1.5 to 2.0 liters were collected in sterile plastic bottles at the plant site from which trucks were loaded. The bottles were tightly sealed, labeled, and stored at 4°C. The next morning, the samples from Medina and Springfield were placed in insulated boxes on ice and shipped to the laboratory via a commercial carrier. The Columbus samples were picked up by laboratory personnel on the day of collection. Attempts to isolate bacteria were done on the day of arrival of the samples to the laboratory.

Stool samples. Stool samples were obtained three times per year both from unexposed controls and from subjects from sludge-receiving farms. The samples were obtained at approximately 4-month intervals with at least one sample obtained during the summer or early fall. The samples were collected in plastic containers which were sealed and either placed in a cooler with ice and delivered to the laboratory the same day or refrigerated overnight and delivered the next day. The samples were tested for bacterial pathogens on the day of arrival.

Human sera. A base-line serum sample was obtained from each subject before the initiation of land application of sludge in a given area. Subsequently, three serum samples per year were obtained from each individual throughout the course of the study. When blood samples were obtained from subjects outside the Columbus area, they were centrifuged on the same day by a local commercial laboratory, and the sera were transferred aseptically to sterile vials and transported on ice to our laboratory.

Isolation of salmonellae and shigellae from sludge. Unless otherwise noted, all media were obtained as dehydrated bases from Difco Laboratories, Detroit, Mich. (Fig. 1).

(i) **Day 1.** Sludge was swabbed directly onto plates of Hektoen Enteric agar (HEK), MacConkey agar (MAC), and xylose-lysine deoxycholate agar (XLD). The plates were then streaked for isolation. Impregnated swabs were added to tubes containing 10 ml of Selenite or GN broths (BBL Microbiology Systems, Cockeysville, Md.). In early samplings, Tetrathionate broth (BBL) tubes were also inoculated. This procedure was later abandoned because recovery was no better than with the other two enrichment media. Plates and enrichment broths were incubated at 37°C for 24 h (plates) or for 24 and 48 h (broths).

(ii) **Day 2.** Colonies producing H_2S were picked from HEK and XLD agars and tested on biochemical media. Lactose-negative colonies were tested for oxidase activity, and those without activity were tested on biochemical media. The enrichment media cultures were streaked on HEK, XLD, and MAC agars and incubated for 18 to 24 h at 37°C.

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The biochemical media used for screening were Triple Sugar Iron agar, lysine-indole agar, and urea agar. Colonies were also suspended in 0.3 ml of saline containing a disk of *o*-nitrophenyl-β-D-galactopyranoside. All tubes were incubated for 18 to 24 h at 37°C.

(iii) Day 3. All plates streaked from the enrichment broths were examined above, and suspected colonies were inoculated into biochemical test media as described above. All tubes of biochemical reactions were examined, and where the reactions indicated the possible presence of salmonellae, API 20E strips (Analytab Products, Plainview, N.Y.) were inoculated according to the directions of the manufacturer. The enrichment cultures were again streaked on HEK, XLD, and MAC plates.

(iv) Day 4. Plates streaked for isolation on day 3 were examined as described above. Biochemical tests and API inoculations again were performed on all suspected colonies. Any colony which was identified as a *Salmonella* sp. was transferred to three Trypticase soy agar (BBL) slants for use in serological identification. All salmonellae isolated were tested by agglutination for group antigens by using group-specific sera (Difco) and then submitted to the Ohio Health Department Laboratory, Columbus, Ohio, for species identification.

Antibiotic sensitivity testing and biochemical confirmation were done in the Clinical Bacteriology Laboratories of The Ohio State University Hospital. All confirmed salmonellae were repurified and freeze-dried in skim milk and stored at −70°C.

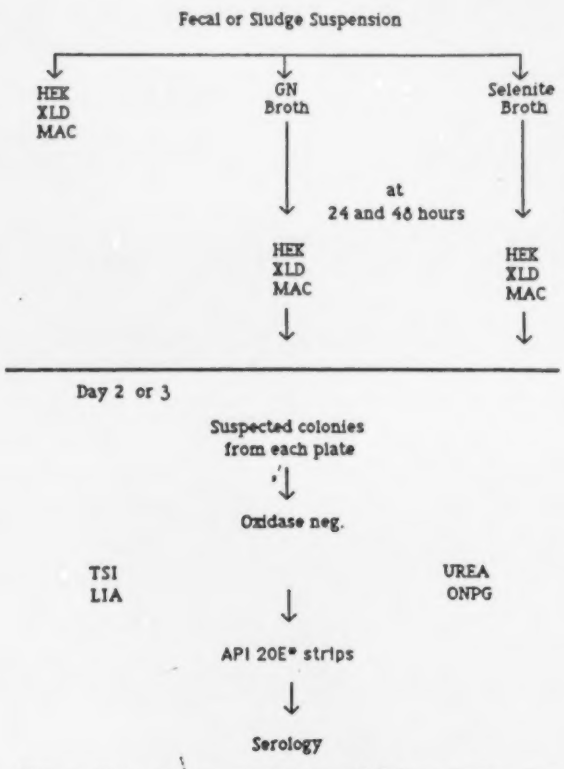

FIG. 1. Flow chart for the isolation of *Salmonella* spp. from wastewater sludge. Abbreviations are as defined in the text. *API is a trademark of Analytab Products.

Isolation of salmonellae and shigellae from stool specimens. A 10% suspension of the stool specimen (less than 24 h old) was cultured and handled as described above for sludge. A total of 1,821 stool samples were tested. When a specimen was positive, repeat cultures were taken every 3 weeks until two consecutive negative cultures were obtained.

Recovery of salmonellae and shigellae from seeded sludge samples. Three different strains of salmonella were used. For the early experiments, a stock of *Salmonella typhimurium* from our laboratory collection was used. For later experiments, strains of *S. typhimurium* and of *Salmonella saintpaul*, both isolated from sludge, were used. A *Shigella flexnerii* strain from our departmental collection was used. Two methods were used to test the effectiveness of our recovery system.

(i) Seeding of samples at plant. Two 1,200-ml samples of sludge from the Medina 300 plant were seeded with 2×10^7 shigellae and 1×10^7 salmonellae to give a final concentration of ca. 1.6×10^4 shigellae per ml and 8×10^3 salmonellae per ml. These samples were then handled as described above for sludge samples.

(ii) Seeding of samples in the laboratory. The salmonella strain to be used was inoculated into three 9-ml samples of freshly received sludge to give final concentrations of ca. 2×10^3, 2×10^2, and 20 CFU/ml. These three samples were then processed together with the routine samples. Experiments were conducted three times with sludge from each site in the study.

Isolation of *Campylobacter* spp. from sludge. For all recovery experiments and for control purposes, we used an isolate of *Campylobacter jejuni* from a patient, which was obtained from the bacteriology laboratories of The Ohio State University Hospital. Sludge was swab inoculated in triplicate on Campy Blood Agar Medium plates (Remel, Lenexa, Kans.) and streaked for isolation. Plates were incubated at 25, 37, and 42°C in stainless steel jars in an atmosphere consisting of 5% O_2, 10% CO_2, and 85% nitrogen. Control plates of *C. jejuni* were included in each jar. The cultures were examined at 24 h and 48 h, and colonies were picked and tested for catalase and oxidase activity. Positive colonies were further tested for motility, H_2S production, and resistance to nalidixic acid and cephalothin.

Recovery of *C. jejuni* from seeded sludge. *C. jejuni* was grown for 48 h on Campy Blood Agar Medium. The organisms were suspended in 3 ml of freshly boiled thioglycolate broth. Tenfold dilutions were made, and calibrated loop counts were done by inoculation in triplicate with a 0.001-ml calibrated loop. Appropriate volumes were inoculated in duplicate into 9 or 9.9 ml of sludge suspension to obtain suspensions ranging from ca. 2×10^6 to 2×10^2 CFU/ml. Mixing of the suspensions was accomplished by bubbling the mixture with the gas used for incubation as described above. The seeded sludge suspensions were tested as described above for the isolation of *C. jejuni*. To test for survival of the organisms in sludge, the suspensions were stored at 4°C for 1 h, 24 h, 4 days, and 7 days before being tested as described above. Because of the viscosity of the sludge obtained from the Columbus Jackson Pike Plant, this sludge was diluted 1:4 with sterile isotonic saline before the addition of bacteria. These experiments also were repeated three times.

Detection of antibodies to salmonella antigens. The last available serum from each individual was tested for antibodies to *Salmonella* groups B, C, D, and E by the rapid slide test by using Bacto-Widal Salmonella O antigens (Difco). Conditions were set so as to run the test at a 1:20 correlated dilution (0.04 ml of serum). Results were scored from

TABLE 1. *Salmonella* recoveries from seeded sludge from the Columbus plant

Final concn (CFU/ml) of added organism	Recovery[a] after incubation for:											
	24 h						7 days					
	HEK	MAC	XLD	HEK[b]	MAC[b]	XLD[b]	HEK	MAC	XLD	HEK[b]	MAC[b]	XLD[b]
0	−	−	−	−/−	−/−	−/−	−	−	−	−/−	−/−	−/−
1.1×10^1	−	+	−	+/+	+/−	−/+	−	−	−	+/+	+/−	+/+
	−	−	−	+/+	+/−	+/+	−	−	−	+/+	+/−	−/−
1.1×10^2	−	−	−	+/−	+/−	+/+	−	−	−	+/+	+/−	−/−
	−	−	−	+/+	+/−	−/+	−	−	−	+/+	+/−	+/−

[a] Appropriate numbers of organisms were added to 10 ml of sludge (in duplicate) and incubated for 24 h or 7 days at 4°C. Isolations were done according to the protocol. −, No salmonellae recovered; +, salmonellae recovered.
[b] From Selenite enrichment broth/GN broth.

negative to 4+, depending on the degree of clumping observed. If the most recently obtained serum of an individual was positive at a level of 1+ or more, the base line (obtained before application of sludge in the area) and subsequent sera were tested until a positive serum was found. If the base-line serum of an individual was also positive for the given group antigen(s), no additional sera were tested.

RESULTS

Recovery of shigellae, salmonellae, and *C. jejuni* from seeded sludge. Two samples of sludge seeded with salmonellae at the plant (Medina 300) were tested. Both yielded the strain seeded (data not shown). Testing of sludge seeded in the laboratory resulted in the detection of the strain inoculated up to 7 days after seeding even when as few as 11 CFU/ml were present. This concentration of organisms was detected only by enrichment techniques. A typical result is shown in Table 1. Direct recovery by plating was seen regularly only when the organisms had been in the sludge for no more than 24 h and were present at 10 times the concentration required for isolation in enrichment medium (data not shown). In one instance when we used for seeding experiments sludge that already contained group C salmonella, both the seeded group B salmonella and the group C salmonella were recovered. The frequency of isolation of salmonella after direct plating or enrichment procedures is shown in Table 2. Twenty-two recoveries from sludge samples and from three stool samples were analyzed to determine on which media the salmonella colonies were detected. In only five cases were the organisms recognized on primary isolation plates. Two were human isolates, and the others were one each from the two Medina plants and the Columbus plant. No shigellae were recovered in our seeding experiments, even when organisms were added to a final concentration of 1.6×10^4 CFU/ml. *Campylobacter* sp. was recovered when ca. 150 CFU/ml were present. Recovery was successful, even when organisms had been held in sludge up to 7 days at 4°C. Results of a typical experiment are shown in Table 3.

TABLE 2. Media which yielded the recovery of *Salmonella* spp. from sludge and human stool specimens

Sample source	Species	Enrichment[a]			
		Primary	GN	Selenite	Tetrathionate
Human	*S. muenchen*	−	−	+	−
Human	*S. java*	+	−	+	−
Medina 300	*S. saint-paul*	+	+	−	−
	S. newington	−	−	+	−
Medina 500	*S. infantis*	−	−	+	−
Zanesville[b]	*S. hinshawii*	−	−	+	−
Columbus	*S. derby*	−	−	+	−
Medina 300	*S. san diego*	−	−	+	−
Human	*S. muenchen*	+	−	+	−
Medina 500	*S. oranienburg*	+	−	−	−
Columbus	Untypable	−	−	−	+
Columbus	*S. agona* (3 isolates)	−	−	+	−
Columbus	*S. infantis*	−	−	+	−
Columbus	Untypable	−	−	+	−
Columbus	*S. infantis*	+	−	+	−
Columbus	*S. anatum*	−	−	+	−
Columbus	*S. thompson*	−	−	+	−
Columbus	*S. ohio*	−	−	+	−
Columbus	*S. enteritidis* (2 isolates)	−	−	+	−
Medina 300	*S. saint-paul*	−	−	+	−
Medina 500	*S. saint-paul*	−	+	−	−
Springfield	*S. infantis*	−	−	+	−

[a] Primary isolation is marked + if recovery was made on HEK, XLD, or MAC plates. If recovery was seen after enrichment in GN, selenite, or tetrathionate broths, the corresponding column is marked.
[b] Because of high cadmium content in the sludge, this plant was dropped from the land application part of the study.

Attempts to isolate shigellae and *Campylobacter* spp. No *Shigella* spp. were isolated from any of the sludge or stool specimens tested. No *Campylobacter* spp. were isolated from 99 samples of sludge tested between September 1980 and June 1981. Of the samples, 41 were from the Columbus plant, 18 were from each of the Medina plants, and 22 were from the Springfield plant.

Isolation of salmonellae from sludge. A total of 50 isolations (16%) of *Salmonella* spp. were made from the 311 samples of sludge tested from all sources. The frequency of isolation varied by year and by site (Table 4). Isolation was most frequent from sludge samples obtained from the Columbus

TABLE 3. *C. jejuni* recovery from seeded sludge from Medina 300 plant[a]

Contact time	No. of positive tests with inoculum (CFU/ml) of[b]:		
	15×10^1	15×10^2	15×10^1
1 h	2	2	2
24 h	2	2	1
24 h[c]	2	2	1
7 days	2	2	1

[a] *C. jejuni* was inoculated in sludge to the final concentration indicated and held for the specified time at 4°C before plating.
[b] Two tests were done for each inoculum level and contact time. No organisms were recovered from sludge to which no organisms had been added.
[c] Subcultured in Campy Thioglycolate broth after holding for 24 h at 4°C.

TABLE 4. Salmonella isolation by quarter of year and by site[a]

Site	Quarter				Total positive/total tested	% Positive
	1	2	3	4		
Medina 300	5/19	3/15	1/13	1/17	10/64	15.6
Medina 500	2/18	2/14	1/13	0/18	5/63	7.9
Columbus	14/37	5/28	3/25	9/36	31/126	25.0
Springfield	1/18	1/12	2/11	0/17	4/58	7.0
Total positive/total tested (% positive)	22/92 (24)	11/69 (16)	7/62 (11)	10/88 (11)	50/311 (16)	

[a] Data are presented as number of positive samples/number of samples tested.

treatment plant (25%) and least frequent from samples from the Springfield plant (7%).

The distribution of isolations from a given plant did not follow any pattern, except that there appeared to be an increased isolation rate during the first quarter of each year (Table 4). An overall chi-square test of the data showed a borderline P value ($0.1 > P > 0.05$), so the data were subjected to an analysis of residuals (11) which indicated that a significant deviation existed in the number of isolations made during the first quarter (2.43, standard deviation). A 2 × 2 chi-square analysis of the isolations made during the first quarter versus those of all other quarters combined yielded a P of <0.02, indicating that substantially more isolations of salmonellae were made during the first quarter than would have been expected.

It appeared that the increase was due primarily to the isolations from the Columbus plant. To test this hypothesis, the first-quarter data from Columbus was tested in a 2 × 4 chi-square test against first-quarter data from the other sites. The overall test gave a P of <0.05. When the first-quarter data from Columbus as shown in Table 4 were compared in a 2 × 2 chi square test with the combined data from the other three quarters from Columbus, a significant difference was also noted ($P = <0.05$).

Twenty-one different serotypes and five untypable strains were isolated from the sludge which was applied to land (Table 5). The most frequently encountered serotype was *Salmonella infantis*, isolated on eight occasions; *S. saint-paul*, *S. typhimurium*, and *S. agona* were the next most frequently isolated serotypes (four times each).

TABLE 5. *Salmonella* serotypes isolated from sludges[a]

Serotype	Frequency	%
S. adelaide	1	2
S. agona	4	8
S. anatum	1	2
S. binza	1	2
S. derby	1	2
S. enteritidis	2	4
S. havana	1	2
S. infantis	8	16
S. java	1	2
S. manhattan	1	2
S. montevideo	3	6
S. newington	1	2
S. ohio	2	4
S. oranienburg	3	6
S. panama	1	2
S. reading	2	4
S. saint-paul	4	8
S. san diego	1	2
S. tennessee	1	2
S. typhimurium	4	8
S. thompson	2	4
Nontypable	5	10

[a] Only isolations from sludges obtained from plants used as source of material applied to land are listed.

Antibiotic sensitivity patterns of the salmonellae isolated from sludge. The antibiotic sensitivity pattern of the salmonella strains isolated is important for epidemiological reasons, and Table 6 presents the distribution of the MICs of given antibiotics for most of the strains isolated from sludge. A few strains were lost before antibiotic sensitivity could be tested. Most strains showed low MICs of most antibiotics. Five strains (10%) showed multiple resistance patterns and are described in Table 7.

Isolation of salmonellae from individuals. The isolations made from individuals are indicated in Table 8. One individual tested positive after repeated cultures. In none of the cases was clinical disease correlated with the isolation of salmonellae from the stool.

Antibodies to salmonellae in the farm population. The serological status of the subject population at the end of the study is shown in Table 9. We were interested in determining possible infection reflected by antibody increases; thus, individuals with no antibodies at the end of the study were assumed to have been negative at the start of the study.

Statistical analysis of the data by the chi-square test indicates that there was a significantly greater ($P \leq 0.001$) incidence of antibodies to group C salmonellae than to the other groups.

DISCUSSION

Enteric bacteria in sludge. The procedure we used for the isolation of salmonellae from sludge is similar to that described by Dudley et al. (9), in that we too used Selenite broth as one of our enrichment media and incubated the enrichment at 37°C rather than at 42°C as suggested by various authors (4, 14, 20, 21). Edgar and Soar (10), in a study comparing methods for the isolation of salmonellae, reported that several methods described in the literature produced different results not only because some of the media used were inhibitory for the organisms but also because, in some instances, some were inhibitory when used at higher incubation temperatures. These authors reported that brilliant green agar was the best plating medium that they tested.

Although we did not use brilliant green as the plating medium, we obtained good growth and differentiation with HEK and XLD plating media, with the former giving better results. The sensitivity of our isolation system was sufficient for our purposes in that we were able to detect 11 CFU/ml of seeded sludge (1.9 to 6.0% solids). Dudley et al. (9) reported a detection limit of >2 to <24 CFU/g of total solids. In studies directed to the determination of the most probable

TABLE 6. Distribution of MICs for *Salmonella* spp. isolated from sludge

Antibiotic	% of isolates[a] at MIC (μg/ml)											
	≤0.5	0.5	≤1	1	≤2	2	4	8	16	32	64	128
Ampicillin	2			40		46	5	2		2		
Carbenicillin[b]					10		80					10
Ticarcillin					28		58	9				5
Cephalothin			2		16	44	30	7				
Cefamandole	25	63		7		5						
Cefoxitin			9		16	28	42	5				
Gentamicin	67			26		2	5					
Tobramycin	37			44		12	7					
Amikacin			35			42	14	9				
Tetracycline			12			70	9				7	
Chloramphenicol							53	40	7	5	2	

[a] n = 43 isolates tested for antibiotic sensitivity. Total isolates, n = 50. Percentages are rounded to nearest integer.
[b] n = 21 isolates.

number of salmonellae in sludges from different sources, Jones et al. (15) were able to find organisms in sludges containing as few as 0.3 salmonellae CFU/100 ml. The method used by Jones and his colleagues (15) was much more extensive than ours in that multiple enrichment systems were used.

As with the results reported by Dudley et al. (9), we also were unable to detect *Shigella* spp. in sludge samples. Our recovery experiments suggest that shigellae do not survive well in sludge. This would decrease the probability that this organism would be present in the sludge applied to land. However, we cannot exclude the possibility that the method used by us and other workers is not sufficiently sensitive to permit isolation. Because of this lack of isolation of *Shigella* spp. from original sludge or from seeded sludge, we are unable to reach any conclusion about the presence or absence of this pathogen or about the relative risks involved.

Our inability to recover *Campylobacter* spp. from samples of sludge despite recovery of the organism from our seeded experiments even after 7 days at 4°C indicates that the organism, if present in sludge, was undetectable by our methods. The high sensitivity of this organism to oxygen might make its presence unlikely in aerobically digested sludges.

The different rates of isolation of salmonellae from the different sites (Table 4) is not surprising. Similar results have been obtained by Danielsson (6), Dudley et al. (9), and Jones et al. (15). The rate of isolation depends on the initial number of salmonellae present in the raw sewage and on the effects of the particular treatment. Our lowest frequency of isolation, 7% from the sludge from Springfield, was similar to the rate of 8% reported by Danielsson (6) in her studies in Sweden.

We are unable to relate the higher number of isolations from the Columbus plant during the first quarter of each year to any specific condition at the plant. Carrington et al. (3) showed that the inactivation of salmonellae in anaerobic sludge was affected by temperature during digestion, so we subjected the frequency of isolation data to correlation analysis, reasoning that in the first quarter of the year, temperatures would tend to be lower and thus the organisms might be protected. Sludge temperature data (not shown) available to us indicated that the temperture of the digestors varied between 30 and 35°C during the course of the study and that isolation of organisms was not correlated with the temperature of the digestors.

To assess the relative risk of infection with salmonella, the concept of infective dose under natural conditions must be considered. It is difficult to establish such a concept because various estimates have been made about the number of viable salmonellae required for infection. Cliver (5) reported that salmonella gastroenteritis occurred in adults and children who had eaten chocolate contaminated with <1 to 100 *Salmonella eastbourne* organisms per 100 g. D'Aoust (7) reported infection doses of 0.7 to 6.1 cells in a series of cases resulting from the ingestion of *S. typhimurium*-contaminated cheese. In another case, Lang et al. (17) reported that 15,000 cells of *Salmonella cubana* were required for illness in patients ingesting contaminated carmine dye. In a study of the naturally occurring infection with *S. typhimurium*-contaminated drinking water in Riverside, Calif., in 1965 (2), it was indicated that the water contained 17 CFU/liter. The

TABLE 7. Multiple resistance to antibiotics in salmonellae isolated from sludge

Source	Serotype	MIC (μg/ml) of:										
		Ampicillin	Carbenicillin	Ticarcillin	Cephalothin	Cefamandole	Cefoxitin	Gentamicin	Tobramycin	Amikacin	Tetracycline	Chloramphenicol
Medina 500	*S. oranienburg*	2	4	≤2	≤2	≤0.5	4	4	4	8	>128	>16
Columbus	*S. infantis*	>32	>128	>128	8	2	8	≤0.5	1	2	>128	8
Columbus	*S. typhimurium*	>32	ND[a]	>128	8	2	2	1	2	4	>128	4
Columbus	*S. montevideo*	8	ND	8	8	1	4	≤0.5	1	≤1	4	16
Springfield	*S. agona*	2	ND	8	4	0.5	4	≤0.5	1	2	4	16

[a] ND, Not done.

TABLE 8. Isolation of salmonellae from human stool specimens tested, 1978 to 1982

Subject no.	Source of sludge	Date	Species
100901	Medina	12/5/78	*S. muenchen*
150602[a]		12/11/78	*S. java*
400406	Columbus	7/24/79	*S. oranienburg*
401704	Columbus	11/18/81	*S. enteritidis*
401704	Columbus	12/3/81	*S. enteritidis*

[a] Non-sludge-receiving farm.

authors of that report, however, advise caution in interpreting their data for the determination of infective dose because of some of the procedures used in sampling the water.

Ayanwale et al. (1) reported feeding goats for 17 months on corn silage grown on land fertilized with sludge from which *Salmonella newport* C_2 had been isolated. No increases in antibodies to the organism were seen. Under controlled experimental conditions, infective doses for *Salmonella* spp. other than *Salmonella typhosa* have been reported to vary between 10^5 and 10^8 cells per host, although in some experiments, 10^6 cells did not cause either disease or infection (summarized by Kowal in reference 16). Because of these considerations, we believe that the effort and expense involved in routinely determining the most probable number of salmonellae in sludge samples is not justified, since no assessment can be made of the potential of a given strain to cause disease in the numbers likely to be consumed.

On the basis of our recovery experiments from seeded sludge samples, the relatively low rate of salmonella isolation, and the fact that most of our samples required enrichment for isolation, it is likely that the number of organisms in the sludges used in this study was usually less than 11 to 40 CFU/ml. When cells were present in higher concentrations, there probably were not more than 280 CFU/ml, since in only a few cases were we able to recover organisms directly from plated material without previous enrichment. These levels of recovery are in line with the findings of Dudley et al. (9).

There is also the concern that the lack of isolation of salmonellae from sludge might not be a sufficient criterion for the assessment of risk since there is always the potential for repopulation of the sludge while it stands before being delivered to the fields. Russ and Yanko (19) reported that in composted sludges (not exactly analogous to conditions of the present study), the pathogen repopulated readily if the moisture content was at least 20%, if the temperture was in the mesophilic range, and if the carbon-nitrogen ratio was in excess of 15:1. The first two conditions existed in our study, but the carbon/nitrogen ratio was well below 15:1 (18).

Serological examination for antibodies to salmonellae. Because agglutinating reactions to salmonella antigens may be a reflection of cross-reactivity with antigens from other members of the *Enterobacteriaceae*, we did not conduct as rigorous a survey of agglutinating antibodies as we did for neutralizing antibody increases for enteroviruses (12). Our method was not designed to detect small increases and decreases in antibodies to the different groups of salmonellae; rather, it detected antibodies on an all-or-none basis with a titer of 1-20 as our base line. By using these criteria, it can be seen that there is a significant difference in the antibody patterns detected in different locations (Table 9). Different families showed distinct serological patterns, possibly showing different patterns of intrafamily spread or common source of infection (Table 10). We did not have enough data for a statistical analysis of any relationship between the occurrence of antibodies to salmonellae and the presence of animals on a farm. Similarly, because of the relatively low number of individuals with antibodies, it was not possible to determine whether there was a significant difference at the end of the trial between the individuals on farms where sludge was used and individuals on control farms. The small number of apparent conversions (Table 11)

TABLE 9. Subjects with agglutinating antibodies to salmonella serotypes

Salmonella serological group	No. of subjects with antibodies in county (total no. of subjects in county)			
	Medina (50)	Franklin (39)	Pickaway (111)	Clark (62)
B	8	0	11	1
C	25	8	14	18
D	8	0	18	7
E	8	1	9	5

TABLE 10. Family patterns of antibodies to salmonella O antigens

Subject[a]	Results for serological group[b]			
	B	C	D	E
100401	+	+	+	−
100402	−	−	+	−
401701	−	−	−	−
401702	−	−	−	−
401703	−	−	−	−
401704[c]	Rise[d]	−	−	Rise
401705	−	−	−	−
100801	−	+	−	−
100802	−	+	+	+
100804	−	Conversion[e]	+	+
100805	−	+	+	+
100806	+	+	−	+

[a] The first four digits of each designation indicate location and farm. The last two digits indicate family members.

[b] +, Agglutination at a serum dilution of 1:20; −, no agglutination seen.

[c] Rises followed infection with *S. enteritidis* (two isolations from stools). No serial dilutions were done, so the negative result with group D antigen might be the result of prozone effect.

[d] Rise. Increase by two grades of reactivity in paired consecutive samples anytime during the study.

[e] Conversion. Serological conversion from negative to positive in paired consecutive samples anytime during the study.

TABLE 11. Conversions and rises

Salmonella serological group	No. of changes in serological reactivity between base line and first serum samples in[a]:	
	Medina County	Pickaway County
B	0	1 rise 2 conversions
C	2 conversions	0
D	0	1 rise
E	0	2 conversions

[a] For definitions of rise and conversion, see Table 10, footnotes *d* and *e*, respectively. For Franklin County and Clark County, no changes were recorded for any serological group. Base-line sera were obtained before any application of sludge to land in the area.

indicated a low risk of infection but precluded a valid statistical analysis.

Thus, it would appear from our studies that the frequency of isolation of *Salmonella* spp. in wastewater sludge varies from plant to plant and season to season and that the risk of infection, as measured by the rapid agglutination test, of the population exposed to such salmonellae is minimal and no different from that of the nonexposed population.

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